Structure of Porcine Secretin. I. Degradation with Trypsin and Thrombin. Sequence of the Tryptic Peptides. The C-Terminal Residue*

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ABSTRACT: Porcine secretin is a basic linear polypeptide with 27 amino acid residues. It contains no lysine but four arginines and one histidine, which is N-terminal. When completely degraded with trypsin three arginyl–leucyl bonds and one arginyl–aspartyl bond are split with the formation of five peptides. Of these peptides one is recognized as the N-terminal part of secretin, because of its histidine content, and the other as the C-terminal part because of the absence of arginine and because it has C-terminal valinamide. If the degradation with trypsin is allowed to proceed for only a short time the arginyl–aspartyl linkage is selectively left largely intact and the peptide Leu-Arg-Asp(Ala,Ser)Arg may be isolated from the digestion mixture. Conversely, on treatment of secretin with thrombin, the Arg-Asp bond is preferentially split with the formation of only two main peptides, the Leu-Arg moiety now being found as part of the histidine containing N-terminal peptide. These data permit the establishment of the sequence of the tryptic peptides in the molecule of porcine secretin.

Porcine secretin was first prepared in a form sufficiently pure for structural analysis in 1961 (Jorpes and Mutt, 1961). It was found to be a heptacosapeptide which on acid hydrolysis yielded the following amino acids in the proportions shown: $Ala_1Arg_4Asp_2Glu_3Gly_2His_1Leu_6Phe_1Ser_4Thr_2Val_1$ (Jorpes *et al.*, 1962).

The only histidine was found to be N-terminal, followed by serine and aspartic acid. Structural work on the polypeptide has been slow because of the small amounts of material available. This has precluded the use of many of the conventional techniques for determination of polypeptide sequence.

Attempts to split the molecule using a commercial preparation of crystalline trypsin led at first to results that were difficult to interpret (Jorpes *et al.*, 1962). When the trypsin used was treated with diphenylcarbamyl chloride to inactivate contaminating chymotrypsin (Erlanger and Edel, 1964) the picture clarified. Five peptides were formed on complete digestion. Four of them contained arginine but one did not. This is consistent with a structure for secretin where the four arginines are in the chain, none terminal, and none in juxtaposition to another arginine.

On paper chromatography of the tryptic digest in a 1-butanol–acetic acid–pyridine–water system (Waley and Watson, 1954) five fractions were obtained. A typical chromatogram is shown in Figure 1. The five fractions are designated a to e according to their position on the chromatogram.

On paper electrophoresis at two pH values, 3.6 and 6.4 (Michl, 1951; Ingram, 1963), fractions a, d, and e were found to be essentially homogeneous. The trace amounts of peptide material discernible in addition to the predominant components were not investigated further. The main components were designated peptides a, d, and e in analogy with the chromatographic fractions. All three of them moved to the cathode at both pH values, peptide d faster than the other two. Peptide e showed a characteristic tendency to spread.

Fractions b and c also showed one main component each but, as could be expected from the chromatographic picture, they were contaminated with one another. Fraction b separated into three components. One component, designated peptide c, gave a negative reaction with the Pauly reagent and migrated swiftly toward the cathode, and a second was the Pauly-positive main component, peptide b, which moved a short distance toward the cathode at pH 3.6 but toward the anode at pH 6.4. A small component that moved a short distance toward the cathode at pH 6.4 was not analyzed. Fraction c was almost entirely composed of peptide c, the only other material present being a small quantity of peptide b and of an unidentified component that moved toward the anode at pH 6.4 slightly faster than the latter.

For amino acid analysis peptides a, d, and e were eluted from the paper chromatograms and, after acid hydrolysis, analyzed qualitatively by the Redfield (1953) method and quantitatively using the Spackman, Moore, and Stein principle (Spackman *et al.*, 1958). Peptides b and c were eluted and, prior to analysis,

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purified further by paper electrophoresis with heavy losses in yield.

Qualitatively it was found that peptide a was hydrolyzed to alanine, arginine, aspartic acid, and serine, peptide c to arginine, glutamic acid, and leucine, peptide d to arginine and leucine, and peptide e to glutamic acid, glycine, leucine, and valine. Quantitation of the ninhydrin color eluted from the paper chromatograms according to Heilmann *et al.* (1957) strongly indicated that the amino acids in each of these four peptides occurred in equimolar amounts with the exception of the leucine of peptide e which seemed to be in threefold excess over the others. These four peptides consequently account for 15 of the 27 amino acids of secretin, and the composition of peptide b should by difference from the composition of secretin be $Arg_1Asp_1Glu_1Gly_1His_1$-$Leu_1Phe_1Ser_3Thr_2$.

The correctness of the picture obtained was supported by quantitative analysis of the tryptic peptides deriving from 1.2 mg (about $^1/_3$ μmole) of secretin.

The results given in nanomoles were as follows, the figures in parentheses giving the assumed number of residues: peptide a: Ala 227 (1), Arg 217 (1), Asp 221 (1), Ser 195 (1); peptide b: Arg 27 (1), Asp 31 (1), Glu 28 (1), Gly 29 (1), His 21 (1), Leu 26 (1), Phe 23 (1), Ser 71 (3), Thr 47 (2); peptide c: Arg 60 (1), Glu 67 (1), Leu 46 (1); peptide d: Arg 85 (1), Leu 83 (1); peptide e: Glu 193 (1), Gly 186 (1), Leu 695 (3), Val 224 (1).

The validity of the results is supported by the fact that the sum of the amino acids of the five peptides gives the amino acid composition of secretin both qualitatively and quantitatively.

These results, and the results from the determination of the N-terminal amino acids of the peptides, may be summarized as follows (based on the assumption that trypsin had split the arginyl linkages): peptide a: Asp(Ala,Ser)Arg; peptide b: His-Ser-Asp(Glu_1Gly_1-$Leu_1Phe_1Ser_2Thr_2$)Arg; peptide c: Leu-Gln-Arg; peptide d: Leu-Arg; peptide e: Leu($Gln_1Gly_1Leu_2$)Val-NH_2. The His-Ser-Asp- sequence in secretin has been known earlier. The number and positions of the amide groups in the individual peptides are based on electrophoretic evidence. Conclusive evidence that valinamide is the C-terminal of peptide e, and therefore of the intact secretin, was obtained by electrophoretic isolation of valinamide as one of the products of degradation of peptide e with "Crystalline Bacterial Proteinase Novo." Its identity was established by cochromatography with an authentic sample of valinamide.

It is obvious that peptide b derives from the amino end and peptide e from the carboxyl end of secretin. (Peptide b is that tryptic peptide that contains the single histidine, which is known to be N-terminal in secretin; peptide e is the only peptide that lacks arginine and also has a C-terminal α-amide.)

When the digestion with trypsin was carried out for only a short time it was found (Figure 2) that, while peptides b, c, and e were formed as before, peptides a and d were formed in much smaller quantities. At the same time a new peptide, peptide a + d appeared. This peptide had the amino acid composition $Ala_1Asp_1Ser_1$-Arg_2Leu_1. The leucine was N-terminal and on prolonged digestion with trypsin the peptide split into peptides a and d. Consequently it had the structure d–a or Leu-Arg-Asp(Ala,Ser)Arg.

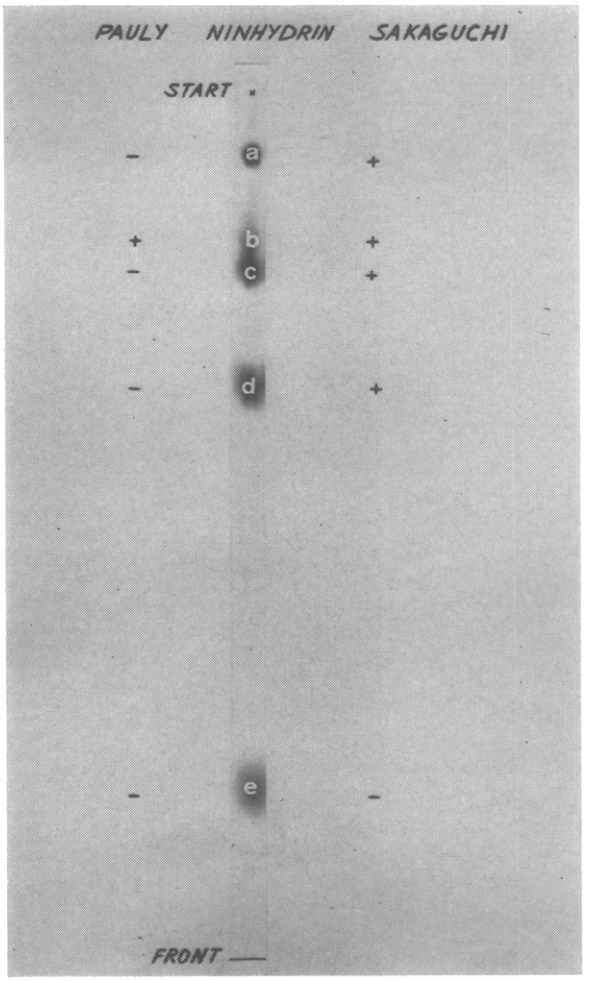

FIGURE 1: Descending chromatography of tryptic hydrolysate of secretin on Whatman No. 42 paper in the system 1-butanol–acetic acid–pyridine–water (30:6:20:24) (Waley and Watson, 1954). Peptides are designated a to e, starting from the origin. Peptide e, which is C-terminal in secretin, contains no arginine. Peptide b is N-terminal and the only one that contains histidine.

The slow splitting by trypsin of the Arg-Asp bond is in accordance with what would be expected from the literature (Li, 1957).

When secretin was digested with thrombin two main peptides, peptides f and g (Figure 3), were formed. These peptides had chromatographic mobilities different from those of any of the tryptic peptides. Only peptide f gave a positive reaction with the Pauly reagent. Since thrombin like trypsin is known to split arginyl and lysyl linkages (Sherry and Troll, 1954; Elmore and Curragh, 1963), it seemed likely that f and g had been formed by the splitting of only one of the four arginyl bonds that were susceptible to trypsin. This proved to

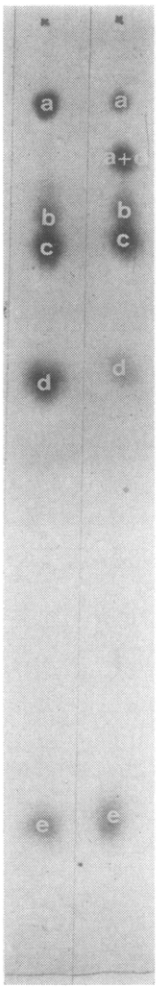

FIGURE 2: Chromatography of secretin. Left: Reference run of tryptic hydrolysate of secretin. Right: chromatogram of secretin after limited digestion with trypsin. Peptides b, c, and e formed as on complete digestion; a and d formed in much smaller amounts. Peptide a + d is on complete digestion split into peptides a and d. Chromatographic conditions as in Figure 1.

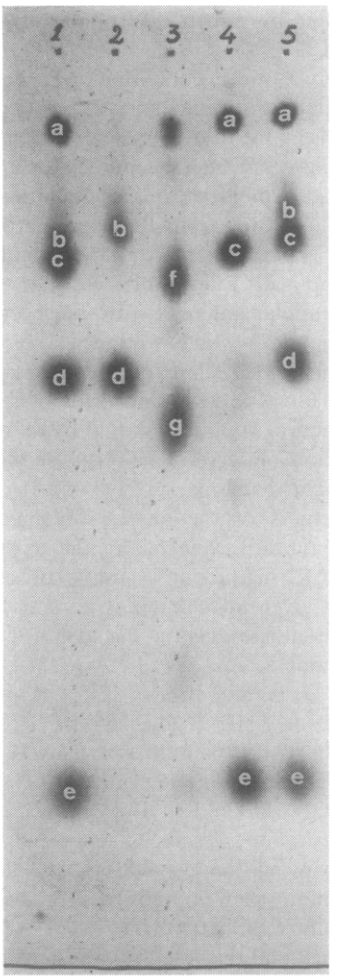

FIGURE 3: Chromatography of secretin. Leads 1 and 5: reference runs of tryptic hydrolysates of secretin. Lead 3: chromatogram of thrombic digest of secretin. If peptide f is digested with trypsin (lead 2) it splits into the tryptic peptide b, which is N-terminal in secretin, and into peptide d, which therefore must be the second tryptic peptide from the N-terminus. Peptide d is known to be linked to peptide a which consequently is the third in order of the tryptic peptides (Figure 2). The thrombic peptide g may be split into the tryptic peptides a, c, and e (lead 4). Chromatographic conditions as in Figure 1.

be the case. When f was digested with trypsin it was split into the tryptic peptides b and d. Peptide g yielded the tryptic peptides a, c, and e. Therefore in secretin the N-terminal tryptic peptide b is linked to peptide d, and since, from the results of the limited degradation with trypsin, d is in turn known to be linked to a, and since e is C-terminal, it is clear that the order from the N-terminus of the tryptic peptides is b = I, d = II, a = III, c = IV, and e = V. This gives the partial primary structure: His-Ser-Asp(Glu_1,Gly_1,Leu_1,Phe_1,Ser_2,-Thr_2)Arg-Leu-Arg-Asp(Ala,Ser)Arg-Leu-Gln-Arg-Leu-(Gln_1,Gly_1,Leu_2)ValNH_2.

It was pointed out earlier that the N-terminal His-Ser- sequence is common for secretin and glucagon (Jorpes *et al.*, 1962). From the above structure additional similarities are evident, namely, the aspartic acid in position 15 and the glutamine in position 20. Work currently in progress in this laboratory on the tryptic peptides a, b, and e indicates that a total of ten amino acid residues occurs in the same position in the two hormones.

Experimental Section

Porcine secretin was prepared according to Jorpes and Mutt; it had the reported activity and amino acid composition (Jorpes and Mutt, 1961; Jorpes *et al.*, 1962). *Bovine thrombin* was prepared according to Magnusson; it had an activity of 1700–2300 NIH units/mg (Magnusson, 1965).

Degradation with Trypsin. Crystalline trypsin (Worthington) was treated with diphenylcarbamyl chloride as described by Erlanger and Edel (Erlanger and Edel, 1964). After centrifugation of the inactivation mixture the clear supernatant was diluted with 1% aqueous ammonium bicarbonate to an approximate trypsin concentration of 2 mg/ml. This solution was stored frozen at −20° and thawed immediately before use.

Complete Tryptic Digestion. Secretin (2 mg) was dissolved in 0.5 ml of water, and 0.5 ml of a 2% aqueous solution of ammonium bicarbonate was added (Katz *et al.*, 1959). The solution turned cloudy. A 20-μl aliquot of the trypsin solution was added every 2 hr and the degradation was allowed to proceed at +21°. Ten minutes after the first addition of trypsin the solution had clarified. After 6 hr it was frozen and lyophilized. The dry residue was dissolved in 1 ml of water and kept on a boiling-water bath for 6 min. It was then frozen and lyophilized again. This technique was also applied for tryptic degradation of the thrombic peptides and for splitting of the arginyl linkage in the peptide Leu-Arg-Asp(Ala,Ser)Arg.

Partial Digestion with Trypsin. Secretin (2 mg) was dissolved in 0.5 ml of water, and 0.5 ml of 2% ammonium bicarbonate was added, followed by 60 μl of the trypsin solution. After 10 min at room temperature the solution was frozen and lyophilized. The residue was taken up in 0.5 ml of water and kept on the boiling water bath for 6 min. It was then frozen and lyophilized.

Digestion with Thrombin. The thrombin stock solution contained 2 mg/ml of thrombin in 1% aqueous ammonium bicarbonate. The conditions of digestion were identical with those used for complete digestion with trypsin.

Paper Chromatography. Descending chromatograms were run at +21° on Whatman No. 42 paper, at right angles to the machine direction of the paper, using the solvent 1-butanol–acetic acid–pyridine–water (30:6:20:24) (Waley and Watson, 1954). A convenient load per spot was the lyophilized tryptic digest from 80 μg of secretin, dissolved in 8 μl of the chromatographic solvent and applied to the paper in aliquots of 2 μl with air-drying between the additions. In preparative runs the spots were spaced on the starting line 0.5 cm apart. After drying the chromatograms at room temperature, guide strips were stained with Pauly and Sakaguchi reagents and with the ninhydrin reagent of Barrollier and co-workers (Heilmann *et al.*, 1957). The color was allowed to develop at room temperature. Dipping, rather than spraying of the paper, was used throughout (Smith, 1960). The peptides were eluted from the papers with 0.1 M AcOH using the apparatus described by Sjövall for quantitative determination of bile acids on paper chromatograms (Sjövall, 1955). The eluates were freeze dried.

Paper electrophoresis was carried out on Schleicher & Schull 2043b paper in aqueous pyridine–acetic acid buffers of pH 3.6 and 6.4 (Michl, 1951; Ingram, 1963). A "cold plate" apparatus (obtained from Savant Instruments, Inc. Hicksville, N. Y.) in which the paper is sandwiched between sheets of plastic supported by a water-cooled metal plate was used for the qualitative runs. When the material was to be eluted for further experiments an all-glass water-cooled apparatus, constructed in this laboratory by Mr. N. Gröndahl, was used instead. The electrophoretic runs were carried out for 2 hr with a voltage gradient of 40 v/cm.

Amino Acid Analysis. Hydrolysis of the peptides was performed with 6 M HCl in an atmosphere of argon at 109 ± 1° for 22 hr.

Qualitative and semiquantitative amino acid analysis was carried out by the method of Redfield (1953) except that Whatman No. 42 paper and the ninhydrin reagent of Barrollier *et al.* (Heilmann *et al.*, 1957) were used. The color was developed at room temperature. The background remained colorless sufficiently long to render optional the recommended steaming of the chromatograms before dipping through the ninhydrin reagent.

Quantitative amino acid analysis was performed by adaptations of the technique of Moore and Stein (Spackman, Moore, and Stein, 1958). At first a one-column micro technique (unpublished) worked out by A. Baldesten was used; later a Technicon Auto-Analyzer was available to us.

N-Terminal amino acids were determined by the phenyl isothiocyanate method of Edman with positive identification of the phenylthiohydantoin derivatives formed. Details of technique and references have been given earlier (Jorpes *et al.*, 1962).

Identification of the C-Terminal Residue of Peptide e (and of Secretin). Peptide e, about 250 nmoles, was dissolved in 0.5 ml of a 1% aqueous solution of ammonium bicarbonate which contained 16 μg of "Chrystalline Bacterial Proteinase Novo" per ml. After standing for 5 hr at 21° the solution was lyophilized and the residue taken up in 0.25 ml of water. This solution was kept for 5 min on the boiling-water bath and then lyophilized.

The residue was subjected to electrophoresis for 1 hr on Schleicher & Schüll No. 2043b paper in a pyridine acetate buffer of pH 6.4 (pyridine–acetic acid–water, 25:1:225 by volume). Glucose was used to ascertain the extent of electroendosmotic flow. The glucose was found to have been carried 2.5 cm toward the cathode. After staining guide strips with the ninhydrin reagent of Barrollier *et al.* an intense canary yellow spot appeared within a few minutes at the same distance from the starting line as the glucose had reached. Another very faint yellow spot appeared 7.5 cm toward the cathode from the starting line. These yellow spots slowly turned orange. (Spots with a transient yellow color are given by the Barrollier type of ninhydrin reagent by many (or all?) peptides with N-terminal glycine or serine.) Considerably later than the yellow spots still another spot, with the usual reddish color, appeared 11 cm toward the cathode and slowly became more and more prominent. An aliquot of the lyophilized eluate corresponding to this spot was hydrolyzed and the hydrolysate examined by paper chromatography in the Redfield system. Only one spot, at the position of valine, was found.

Cochromatography of another aliquot of the hydrolysate with authentic valine gave only one spot. These results suggested that the material eluted from the paper was valinamide. This was confirmed by descending chromatography of an aliquot of the unhydrolyzed eluate material on Whatman No. 42 paper in parallel with valine, valinamide, and valine methyl ester, in the upper phase of the system 1-BuOH–AcOH–H_2O, 25:6:25. (This system was recommended by the Yeda Research and Development, Rehovoth, Israel, from whom the valinamide was obtained. The valine and valine methyl ester were obtained from Fluka A-G, Buchs, Switzerland.)

It was found that under the conditions of the experiment valine had an R_f value of 0.42, valine methyl ester 0.51, and valinamide and the eluate material both 0.34. In another experiment the eluate material and valinamide were cochromatographed. Only one spot was found.

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References

Elmore, D. T., and Curragh, E. F. (1963), *Biochem. J. 86*, 9P.

Erlanger, B. F., and Edel, F. (1964), *Biochemistry 3*, 347.

Heilmann, J., Barrollier, J., and Watzke, E. (1957), *Hoppe-Seylers Z. physiol Chem. 309*, 219.

Ingram, V. M. (1963), *Methods Enzymol. 3*, 885.

Jorpes, J. E., and Mutt, V. (1961), *Acta Chem. Scand. 15*, 1790.

Jorpes, J. E., Mutt, V., Magnusson, S., and Steele, B. B. (1962), *Biochem. Biophys. Res. Commun. 9*, 275.

Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem. 234*, 2897.

Li, C. H. (1957), *Advan. Protein Chem., 12*, 281.

Magnusson, S. (1965), *Arkiv Kemi 24*, 349.

Michl, H. (1951), *Monatsh. Chem. 82*, 489.

Redfield, R. R. (1953), *Biochim. Biophys. Acta 10*, 344.

Sherry, S., and Troll, W. (1954), *J. Biol. Chem. 208*, 95.

Sjövall, J. (1955), *Arkiv Kemi 8*, 317.

Smith, I. (1960), Chromatographic and Electrophoretic Techniques, Vol. I, New York, Wiley.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Waley, S. G., and Watson, J. (1954), *Biochem. J. 57*, 529.